

8-10-1994

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C. A. Bader
Hôpital Necker, Paris

A. Chevalier
Hôpital Necker, Paris

C. Hennequin
Hôpital Necker, Paris

P. Jungers
Hôpital Necker, Paris

M. Daudon
Hôpital Necker, Paris

See next page for additional authors

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Recommended Citation

Bader, C. A.; Chevalier, A.; Hennequin, C.; Jungers, P.; Daudon, M.; Beaugendre, O.; and Hoarau, M. P. (1994) "Methodological Aspects of Spontaneous Crystalluria Studies in Calcium Stone Formers," *Scanning Microscopy*: Vol. 8 : No. 2 , Article 8.

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Authors

C. A. Bader, A. Chevalier, C. Hennequin, P. Jungers, M. Daudon, O. Beaugendre, and M. P. Hoarau

METHODOLOGICAL ASPECTS OF SPONTANEOUS CRYSTALLURIA STUDIES IN CALCIUM STONE FORMERS

C.A. Bader, A. Chevalier, C. Hennequin, P. Jungers and M. Daudon*
with the technical collaboration of O. Beaugendre and M.P. Hoarau

INSERM U 90 and Département de Néphrologie
Hôpital Necker 161 rue de Sèvres 75743 Paris Cedex 15 France

(Received for publication April 18, 1994, and in revised form August 10, 1994)

Abstract

Despite nearly a half-century of study, the clinical value of spontaneous crystalluria (Cx) examinations in calcium stone formers (CaSF) is still uncertain. The analytical complexity of urine particle study is largely responsible for this situation. As a result, there is no consensus regarding technical methods in Cx with several techniques for urine sampling and three different instruments currently used for particle study, namely, particle counting (PC), light microscopy (LM) and petrographic microscopy (PM). In this work, we first examined urine sampling and instrument methods regarding their appropriateness for Cx studies. Then we performed a comparative analysis of Cx studies in CaSF. Despite many technical and clinical discrepancies, several studies agree that the frequency of "all particles" and of the weddellite and whewellite calcium oxalate (CaOx) crystalline phases are increased in CaSF as compared to normal subjects (NS). Particle sizes and aggregation ratio are also often increased. Altogether, these results reinforce the need for an efficient method for Cx studies in these patients. Examining each technique leads us to conclude that most particle parameters can be studied by "direct LM" observation of freshly voided urine samples, i.e., urine samples without any separation steps. For clinical applications, several examinations should be performed, first to define the specific Cx characteristics in a patient, then for the study of treatment efficiency on Cx control, and finally, during the patient follow-up. Due to Cx variability in each patient, the frequency of Cx examinations during each phase needs to be determined in long-term comparative prospective studies of CaSF.

Key Words: Calcium stone formers, crystalluria, urine sampling, particles counting, petrography, light microscopy, patient follow-up.

*Address for correspondence:
M. Daudon,
address as above

Telephone number: 33 1 45 66 04 58
FAX number: 33 1 45 66 51 33

Introduction

The annual incidence of calcium oxalate urolithiasis in western societies ranges between 7 and 21 per 10,000 (Smith, 1989). Consequently, medical and socio-economic consequences of urolithiasis call for new strategies for its prevention (Consensus conference, 1988). Among these strategies, the finding of crystalline particles¹ in urine of calcium stone formers (CaSF) long ago raised strong hopes for the potential usefulness of crystalluria (Cx) examinations in these patients: From "the most ancient times there was a persuasive belief that Cx and stone formation are somehow related" (Hallson, 1988). It was, therefore, expected that Cx studies could give information about the calcium lithiasis processes at work in CaSF: "Since the crystals observed in urine have grown in the urinary tract, they should be subject to at least some of the same factors involved in urolithiasis" (Werness *et al.*, 1981). Finally, hopes that Cx studies could be of diagnostic value, in particular for the prediction of stone recurrence and for patient follow-up, were the greatest incentives of almost one-half century of Cx research in CaSF (Black, 1945). Nevertheless, the interest of Cx studies in these patients is still not firmly established. Most disturbing from the beginning of these studies were the observations that Cx episodes could be observed both in CaSF and in healthy subjects in whom no renal stone disease had been recognized (Cottet and Vittu, 1952; Robertson, 1969; Robertson *et al.*, 1969), showing that there is no "one-to-one" correspondence between Cx and stone formation processes (Werness *et al.*, 1981). The main difficulties encountered during Cx studies, however, are a series of technical and instrumental problems related to both urine sampling and processing, and to the instruments used for particle studies. In this paper, we analyzed these difficulties, the methods developed to solve them, and the results of Cx examinations in CaSF. We finally suggest that "direct light microscopy (LM)" study of urine samples is a simple, reliable and efficient method for Cx studies.

¹The term "particle(s)" is used to cover both crystals and aggregates when a distinction is not relevant.

Figure 1 (a-h on the facing page 217; i-p on page 218). Light microscopic illustrations of calcium oxalate crystals, crystal aggregates and associations most frequently found in urine of CaSF. **From top to bottom and clockwise.** **1a.** Whewellite in oval crystals with swollen extremities ("dumb-bell"). **1b.** Whewellite in pseudohexagonal habit dispersed among a background of granulations made of amorphous complex urates (polarized light). **1c.** Whewellite aggregates of pseudohexagonal crystals with granulations of amorphous complex urates. **1d.** Whewellite in "lenticular" or "hematiform" habit associated with brushite aggregates (arrow) and amorphous carbonated calcium phosphates (ACCP) granulations. **1e.** Whewellite in sticks with straight or slightly swollen sides in a background of amorphous complex urates (polarized light). **1f.** Whewellite in hexagonal crystals with a stretched appearance encountered during ethylene glycol intoxication (polarized light). **1g.** Weddellite in bipyramid crystals ("envelope"). **1h.** Weddellite bipyramid crystals in aggregates with 2 macles. Bars = 16 μm (for 1a to 1f, and 1h) and 65 μm (for 1g).

Particle parameters

Cx studies primarily involve the qualitative and quantitative study of urinary particles using a range of appropriate parameters. These parameters are briefly examined below.

Particle qualitative parameters

Crystalline phases: In aqueous solutions supersaturated with CaOx, crystals corresponding to the three hydrated CaOx salts are observed. In urine, which is normally supersaturated with all three hydrates (Gardner, 1975), crystalline particles corresponding to monohydrate salt, or whewellite, and to dihydrate salt, or weddellite, are observed. CaOx trihydrate, the least stable and most soluble phase, which may occur transiently at the time of initial precipitation (Tomazic and Nancollas, 1979), is found in urine in rare situations such as after certain drug treatments (Daudon *et al.*, 1987b). In addition to the CaOx crystalline phases, several others, including uric acid and urate salts, calcium phosphates and struvite, are observed in urine of CaSF.

Crystalline phase habits: *In vivo*, as in chemical solutions, each crystalline phase can be present under several habits since growth conditions are not necessarily the same for all sides of the crystal (Nancollas, 1979, 1983). The presence of particles with the same crystalline molecular phase but with different habits can lead, if unknown, to the risk of Cx misinterpretations (Osborne *et al.*, 1986). In a few cases, specific crystal habits have been related to particular situations such as the dodecahedral habit of weddellite crystals observed in highly hypercalciuric urine samples in place of the common octahedral habit (Daudon, 1987a) and the whewellite habit observed after ethylene glycol poisoning with a habit strikingly different from the usual oval or dumb-bell habits (Godolphin *et al.*, 1980; Miller, 1990; Terlinsky *et al.*, 1980, 1981).

Aggregates: Due to physico-chemical interactions, crystals can join together in polycrystalline structures or "aggregates". These formations can include various

numbers of crystals and be composed of one or several crystalline phases.

Particle morphology: Isolated urinary crystals and aggregates are frequently identified using either LM (Baumann, 1978; Coe *et al.*, 1992; Elliot *et al.*, 1976; Osborne *et al.*, 1986) or scanning electron microscopy (SEM) (Berg *et al.*, 1976; Edyvane *et al.*, 1987; Khan and Hackett, 1986; Rodgers *et al.*, 1988; Werness *et al.*, 1981). However, in clinical practice, urinary particles are mostly found in various associations such as whewellite and weddellite, weddellite and uric acid, weddellite and brushite, and amorphous calcium phosphate, etc., which are rarely illustrated (Daudon, 1989, 1993; Valyasevi and Dhanamitta, 1974). Other qualitative observations which may be of interest for Cx interpretation concern the presence of heterogeneous nucleation processes observed on epithelial linings, cell debris, urinary casts, etc. (Coe *et al.*, 1992). The main particle habits, their associations and some frequently encountered nucleations in urine of CaSF are illustrated in Figures 1 and 2.

Particle quantitative parameters

Sizing: Particles in urine are present in a wide range of sizes and are not easily measurable (see Figs. 1 and 2). To obtain a representation of the particle sizes in a urine sample, several indicators such as the mean particle size, the maximal particle size, and the size-number distribution spectrum are used.

Abundance: Cx abundance can be quantified using the particle number and volume.

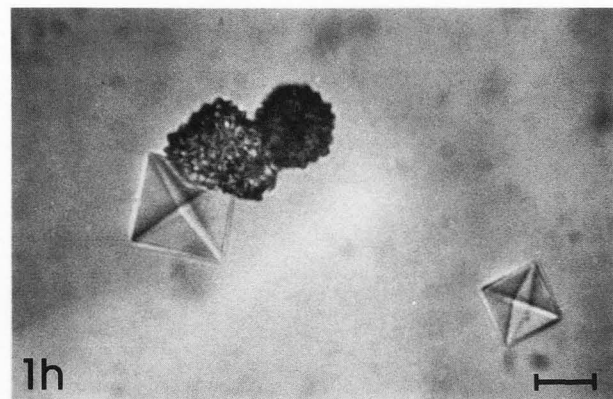
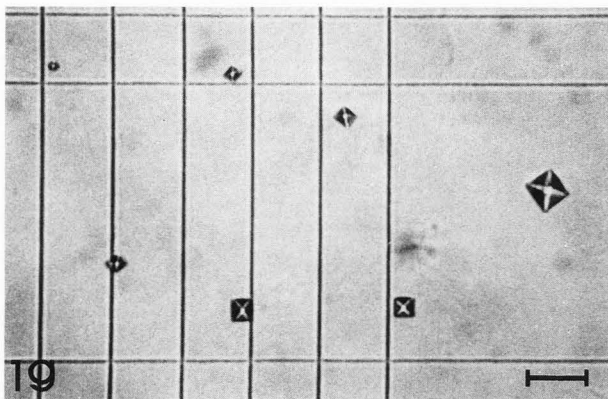
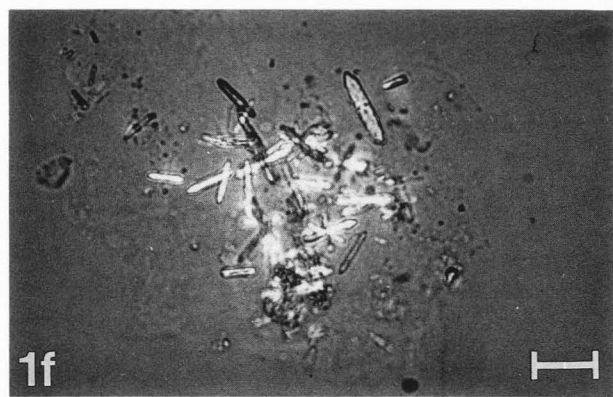
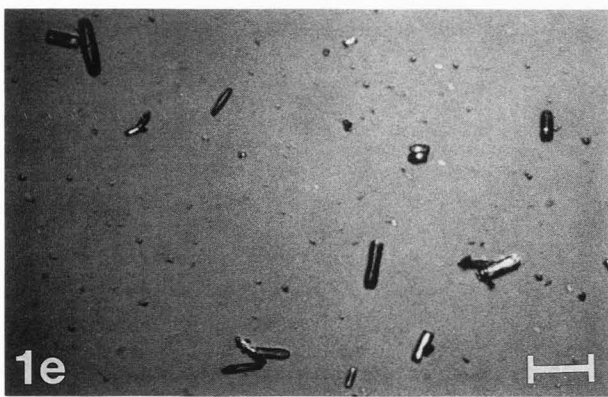
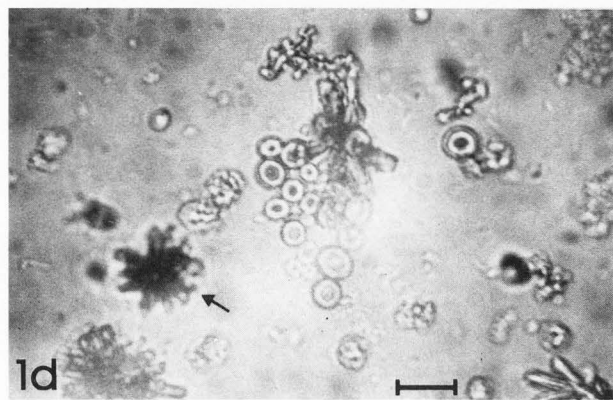
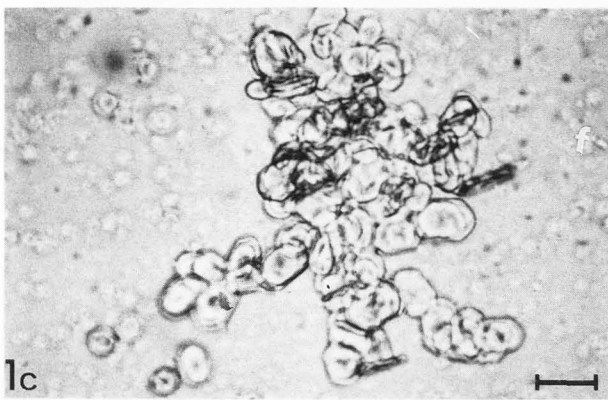
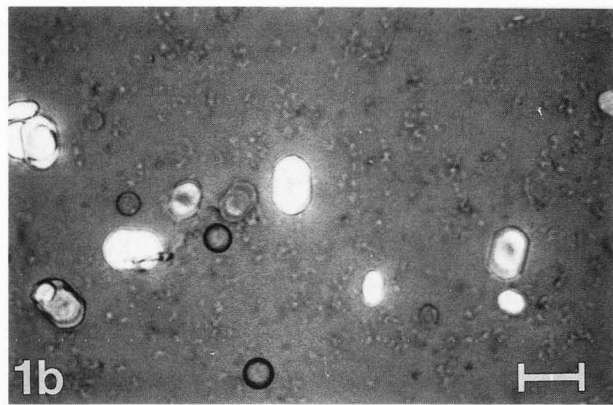
Aggregation ratio

An aggregation ratio is computed as the number of aggregates divided by the total number of particles in a sample.

Particle frequency

Particle frequency relates to the mere presence or absence of particle(s) in a urine sample. Theoretically,

Methods for crystalluria studies in calcium stone formers



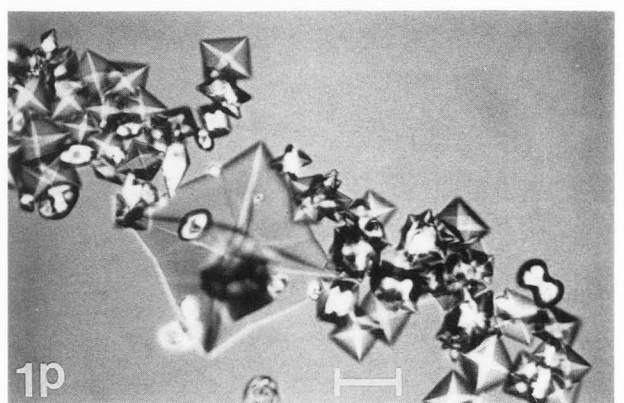
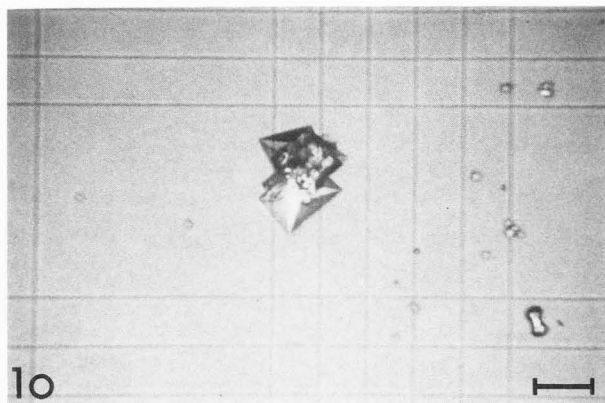
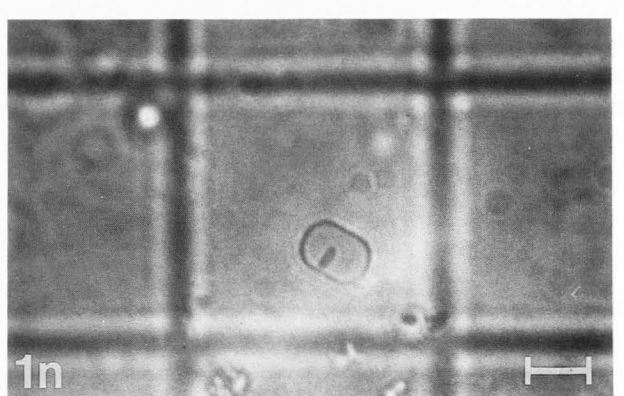
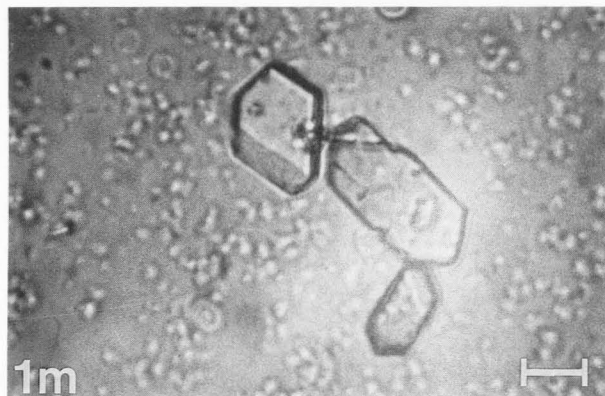
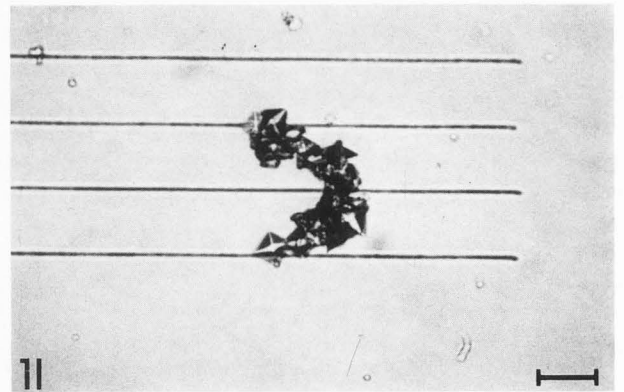
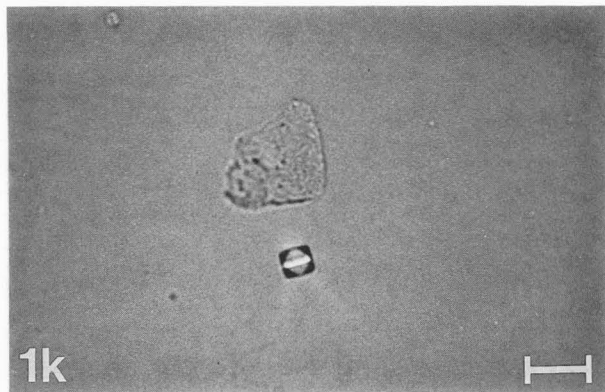
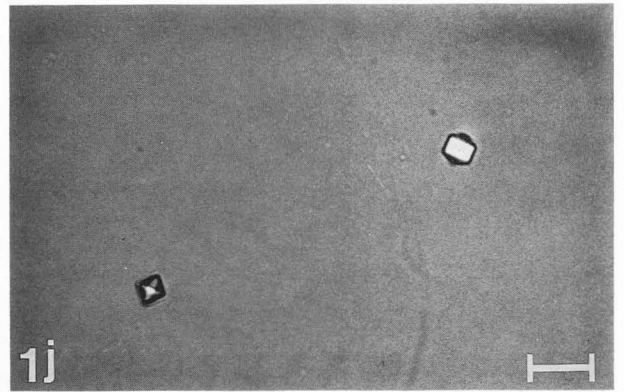
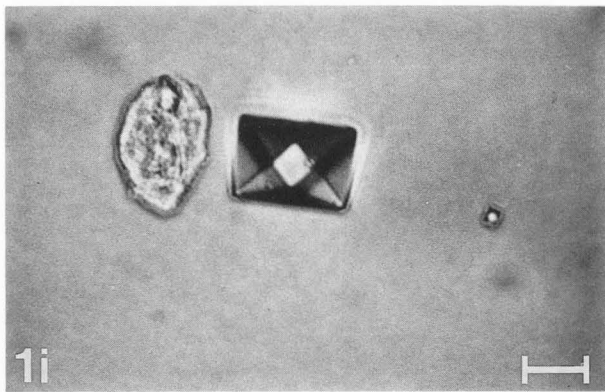


Figure 1 (legend continued from page 216). From top to bottom and clockwise. **1i.** Weddellite in truncated pyramids with a rectangular-deformed base. **1j.** Weddellite in dodecahedral faces giving an hexagonal appearance usually associated with hypercalciuria (Ca > 6 mmol/l) (polarized light). **1k.** Weddellite crystal in square habit with two opposite triangles at the inside (polarized light). **1l.** Large weddellite aggregate composed of about 50 crystals. **1m.** Trihydrated calcium oxalate asymmetric hexagonal habit in thin lamella. **1n.** Trihydrated calcium oxalate in thick plate ("race course"). **1o.** Mixed crystalluria of whewellite crystals in "dumb-bell" and octahedral weddellite crystals in a voluminous aggregate (polarized light). **1p.** Whewellite (oval) and weddellite (envelope) in a voluminous heterogeneous aggregate (polarized light). Bars = 16 μm (for 1i to 1n, and 1p) and 65 μm (for 1o).

Table 1. Crystalluria study. Main crystal qualitative and quantitative variables and associated urine biochemical and physico-chemical variables.

Crystal qualitative variables	Crystal quantitative variables	Urine biochemical variables	Urine physico-chemical variables
crystal species	size; volume; number	calcium; oxalate	pH; density; ionic strength
crystal habits	aggregates number	phosphate; urate	conductivity
heterogeneous nucleation phenomena	aggregation ratio	citrate; sulfate; sodium; potassium; magnesium; pyrophosphate; urea; creatinine	supersaturation
associated cells; cell debris bacteria; casts	frequency	macromolecular inhibitors	crystallization inhibition power

the observation of a single particle is sufficient to qualify an urine sample as "Cx positive". It has long been known that, even in recurrent CaSF, urine samples are not always "Cx positive" (Cottet and Vittu, 1952; Robertson *et al.*, 1969). Conversely, urine samples from normal subjects (NS), i.e., people without any known stone and/or renal disease, are not always "Cx negative", i.e., free of particles. These observations can be quantified using the frequency parameter, i.e., the number of "Cx positive" samples over the total number of samples in a series. Frequency can be studied for the presence of particles in urine whatever the chemical composition giving an "all particles" frequency. Frequency can also be studied separately for each crystalline phase, for example, for the weddellite phase, the brushite phase, etc., as well as for association, such as urate and weddellite phases.

Other crystalluria parameters

To fully describe a urine sample regarding its crystallization status, study of urine particles is necessary but not sufficient. Other urine variables must be used, in particular, the biochemical variables as well as physico-chemical variables such as supersaturation (Robertson *et al.*, 1976a, 1981; Tiselius, 1991; Werness *et al.*, 1985) which, in the final analysis, are responsible for crystallization. All these variables are of great help for the final step of Cx interpretation (Daudon, 1987b, 1993;

Robertson *et al.*, 1981). The main Cx particles and urine associated variables are grouped in Table 1.

Instruments for Particle Studies

The instruments used for clinical application of Cx belong to three different families: light microscopy (LM), petrographic microscopy (PM) and particle counting (PC). For each instrument, its principle, the sample preparation method, and the particle parameters which can be studied are discussed below. The heading **Feasibility** covers the skills required by operators, time per sample, and the associated costs.

Light microscopy (LM)

Principle: Observation is made by optical light transmission. To improve differentiation between crystal habits, polarized light observation was introduced for particle identification (Dyer and Nordin, 1967).

Sample preparation: In most LM studies, "indirect LM" is performed on a 10-20 μl aliquot of the sediment obtained after isolation of the particles by filtration or centrifugation of a 1-2 ml aliquot of the urine sample. "Indirect LM" on sieved fractions of urine were also used in the past (von Sengbusch and Timmermann, 1957a,b, 1958). In our laboratory, we developed "direct LM" studies of urinary samples. In this method, the particles are studied as voided using a 20 μl aliquot of

Figure 2. Mixed crystalluria in CaSF. **From top to bottom and clockwise.** **2a.** Whewellite (oval) crystals associated with complex amorphous urates in granulations (polarized light); **2b.** Heterogeneous crystalluria with whewellite, weddellite (macle) and dihydrated uric acid (arrows) (polarized light). **2c.** Heterogeneous nucleation of octahedral weddellite crystals (arrows) on anhydrous uric acid (polarized light). **2d.** Heterogeneous nucleation of whewellite in stick crystals on anhydrous uric acid crystals (polarized light). **2e.** Heterogeneous nucleation of brushite (arrows) on dodecahedral weddellite with amorphous calcium phosphates in granulations. **2f.** Heterogeneous nucleation of brushite over weddellite and, conversely, of weddellite over brushite. **2g and 2h.** Heterogeneous crystalluria with whewellite, struvite and granulations of amorphous calcium phosphate (**2g**), and with weddellite, struvite and granulations of amorphous calcium phosphate (**2h**). Bars = 30 μm (for 2a, 2b, 2d, 2g, and 2h); 65 μm (for 2c and 2e); and 16 μm (for 2f).

a well-homogenized urine placed in a Mallassez cell as for cytological studies (Daudon, 1987a, 1989; Daudon *et al.*, 1983, 1987a; Nguyen *et al.*, 1987).

Particle study: Qualitative parameters: Using LM, all particle qualitative descriptors can be studied: crystalline phases and habits, aggregates, etc.

Particle study: Quantitative parameters:

Sizing: Sizes of particles are studied using a graduated eye-piece ocular and are limited only by the resolution limit of LM, i.e., 1 μm .

Particle abundance: The total particle number and concentration are measured by taking into account the urine sample and cell chamber volumes. Particle volume is not usually measured by LM although an approximation can be obtained in certain cases (see Discussion).

Aggregation: Composition and number of aggregates can be recorded. From the total particle number and the aggregate number, the aggregation ratio can be computed.

Feasibility: A well-trained technician is able to reliably and rapidly recognize most crystalline phases in their various habits. An extensive qualitative and quantitative "direct LM" Cx study is performed by an experienced observer in about 15-20 minutes. This time must be at least be doubled when using "indirect LM", due to the required technical handling of the sample. A good-quality polarized light microscope with several magnification lenses costs about \$12,000; when equipped for microphotography and video imaging, the cost totals about \$20,000.

Petrography microscopy (PM)

Principle: PM performs crystalline phase determination by comparing the refraction index of the particles with the known refraction indices of organic oils.

Sample preparation: PM requires full isolation of the particles using several filtration and drying steps.

Particle study: Qualitative variables: With PM, the crystalline chemical phases of the main particle can

be studied. The characteristic refractive indices are associated with a given crystalline phase.

Particle study: Quantitative variables:

Particle number: Following the work of Werness *et al.* (1981) semi-quantitative results are obtained using a scale based on the observed number of crystals per filter from a 2 ml sample. A scale is used to attribute a score for quantifying the particles collected on the filters: 0 crystals (i.e., "negative" crystalluria) = 0; 1-200 = 1+; 200-500 = 2+; > 500 = 3+. Summing the scores from the individual voidings and dividing the obtained value by the number of voidings during the 24-hour collection period provides the "crystalluria score".

Particle sizes: These parameters are not usually studied in Cx studies with PM.

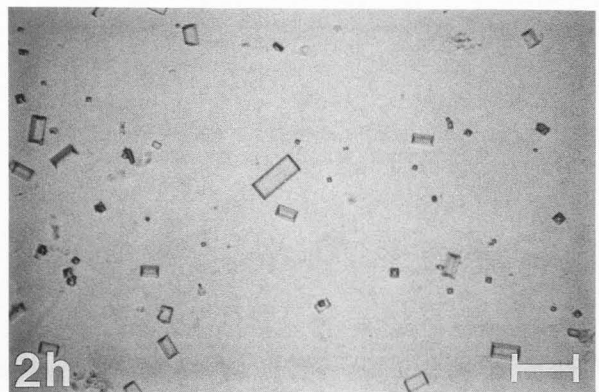
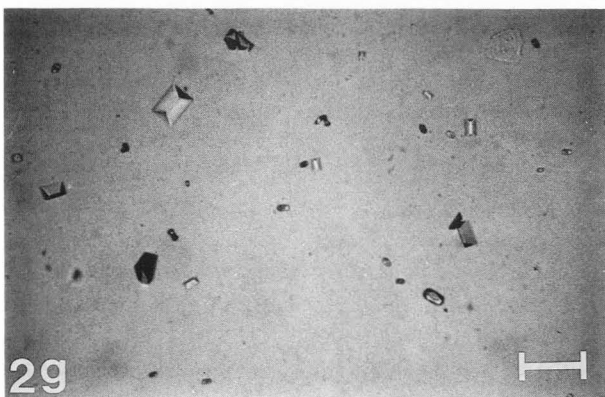
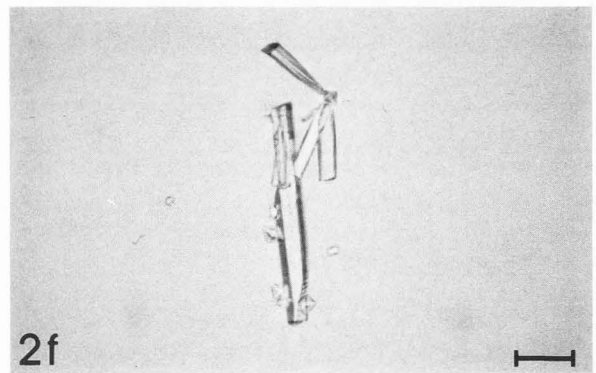
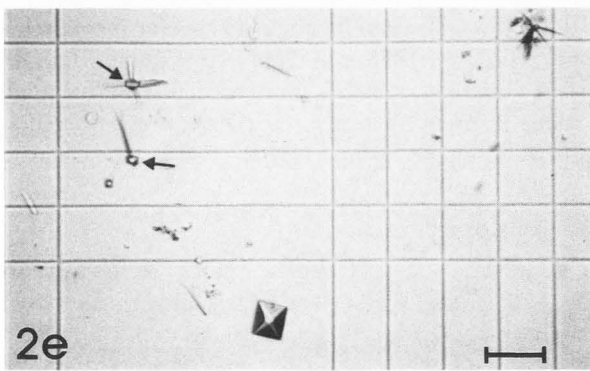
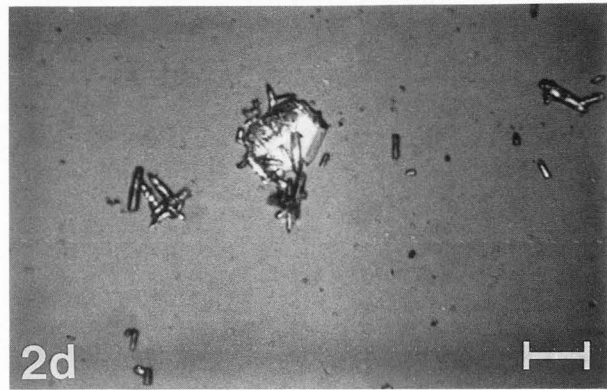
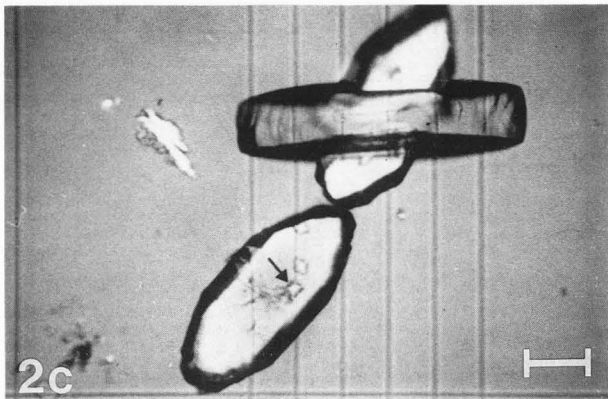
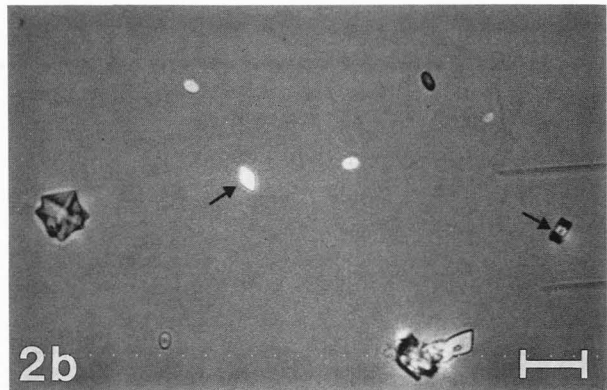
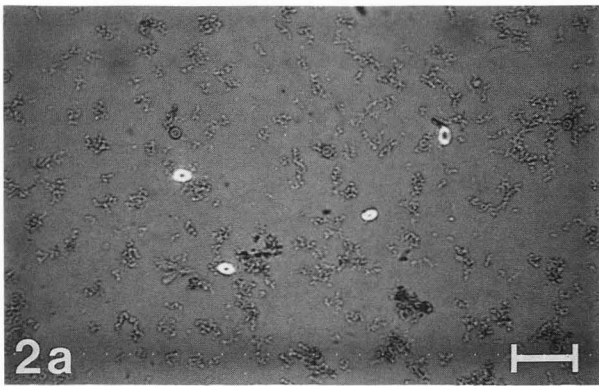
Feasibility: PM requires full isolation of the particles involving lengthy preparation steps. PM observation is a relatively sophisticated method and requires expert technicians. The costs of PM instruments are similar to those for LM.

Particle counting (PC)

Principle: The principle of these instruments is based on a modification of an electric parameter triggered by particles coming across the detector chamber.

Sample preparation: Use of PC in Cx studies was introduced by Robertson (1969). A prefiltration step of the samples is needed to eliminate particles with sizes greater than 74 μm which would plug the inlet of the instrument. Furthermore, the particles have to be put in suspension in an electrolyte. Since every particle which passes across the measuring cell will be recorded irrespective of its nature, a technique for special handling of sample has been developed for counting the CaOx particles only. Two samples are run in parallel: one is ethylenediaminetetraacetic acid (EDTA) treated to dissolve the calcium oxalate and phosphate crystals; its companion sample is similarly treated except that the EDTA is replaced with a saline solution. The difference between the two counts is taken as the neat calcium

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oxalate crystal number.

Particle parameters: Qualitative variables: No direct information regarding the particle can be obtained using PC.

Particle study: Quantitative variables:

Particle sizing: Particles above 3.8 μm were recorded by Robertson (1969), although a smaller size (2.5 μm) has also been recently observed (Rodgers *et al.*, 1991).

Particle quantitation: A size-number distribution spectrum is recorded over the range 3.8-48.6 μm . Standardization of the apparatus permits derivation of a volume-size distribution spectrum. Then, a crystal volume per unit volume of urine or "volume-concentration" is computed.

Feasibility: PC are easy to use and can be equipped with a data treatment facility. Although the instrumental counting step is rapid, sample manipulations require a long time. Cost of instruments is from about \$15,000 to 30,000 depending on automation and computing facilities.

Other instruments for particle studies

Physical methods must be used for the study of crystals less than 1 μm as for most calcium phosphate crystals (Tozuka *et al.*, 1987). Definite identification of the major habits of urinary CaOx crystals has also been made using crystallographic analysis (Catalina and Cifuentes, 1970a,b). These methods are also used for unusual crystal habits, e.g., thin needles seen in urine after ethylene glycol poisoning which were first assumed to be hippurate crystals but were later identified, thanks to X-ray diffraction, as whewellite crystals (Godolphin *et al.*, 1980). SEM, with its large depth of focus, shows the morphological features of crystals and has been used for illustrating CaOx crystals in urine in many studies. X-ray diffraction crystallography, and SEM alone or coupled with an energy dispersive X-ray microanalysis systems, are also sometimes used in clinical studies. In our laboratory, we showed that infrared spectrophotometry alone or coupled to an infrared microscope for individual crystals with sizes above 8 μm allows rapid identification of molecular phases (Daudon *et al.*, 1991). Chemical methods have also been devised for calcium oxalate and phosphate crystal determination in urine (Hallson and Rose, 1988).

Urine Sampling and Processing

Urine biochemical composition and saturation, i.e., the crystallization "driving forces", are highly variable (Finch *et al.*, 1981; Robertson and Peacock, 1984; Robertson *et al.*, 1972, 1976a). As a result, Cx varies

with diet (Cottet and Vittu, 1952), beverages consumed, drinking habits (Black, 1945), and volume of liquid intake (Black, 1945; Berlyne *et al.*, 1976). The post-prandial alkaline tide may favor formation of calcium phosphate nuclei on which calcium oxalate can crystallize (Schwille, 1985). Many other variables, such as, seasons (Hallson and Rose, 1977), physical activity (Irvine *et al.*, 1986; Rodgers *et al.*, 1988, 1991), and ethnic group (Rodgers *et al.*, 1992; Valyasevi and Dhanamitta, 1974) can influence Cx. Another source of Cx variability is urine instability with particles in equilibrium with a supersaturated liquid phase. As soon as the urine is voided, any change in temperature and composition could easily alter this equilibrium with risks for the native particles to be modified qualitatively and quantitatively. Thus, in order to perform reliable comparisons between Cx data, a sampling protocol able to control both for patient variability and urine instability has to be chosen.

Urine sampling

In order to control for patient variability, most primary studies were performed with strict control of patient conditions (Robertson *et al.*, 1969). Patient preparations with regimen loaded in oxalate, calcium and carbohydrates were also used while allowing metabolic lithiasic dysfunction to be revealed by inducing or increasing Cx in these subjects (Cottet and Vittu, 1952, 1953; Robertson and Peacock, 1972; von Sengbusch and Timmermann, 1958). In more recent studies, first urine of the morning, which is more concentrated due to night water restriction, is favored (Daudon and Réveillaud, 1984; Daudon *et al.*, 1983, 1987a; Hermann and Schwille, 1992; Sriboonlue *et al.*, 1990).

Urine storage

In the context of clinical studies, a storage period between patient voiding and examination is generally unavoidable. There is no consensus as to the duration and temperature which would entail no significant modification of the spontaneous Cx properties. In many studies, great care is taken to avoid any temperature variations from the time of voiding to the time of examination, by collecting urine at 37°C and conducting all the technical processes and instrument examination at this temperature. These conditions are not easy to comply with in routine clinical practice and several laboratories questioned the stringency of these conditions. Several authors estimated that urine samples stored for up to 2 hours showed no significant change in urine composition or bacterial effects on crystallization (Hallson and Rose, 1977). Recording samples pH just after voiding, and then at the time of study, allows one to record any changes due to bacteria (Daudon *et al.*, 1983). In these conditions, no changes in mean particle sizes of samples

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Table 2. Frequency of "all particles" and CaOx particles in CaSF and NS.

Reference	Method	Number of subjects CaSF/NS	Frequency % "all particle" CaSF/NS	Frequency % CaOx phases ¹ CaSF/NS
Cottet and Vittu (1952)	"indirect LM"	74/24	78.4/16.7	60/-
Hallson and Rose (1976)	"indirect LM"	121/54	48/22	16/11
Baumann <i>et al.</i> (1984)	"indirect LM"	12/16	-	75/12
Sriboonlue <i>et al.</i> (1990)	"indirect LM"	29/36	68/43	-
Werness <i>et al.</i> (1981)	PM	89/16	100/100 ²	45/18
Caudarella <i>et al.</i> (1986)	PM	86/40	-	78/19.8
Klepikov <i>et al.</i> (1991)	"indirect LM"	372/-	44/17	25.8/12.2
Chevalier <i>et al.</i> (unpublished)	"direct LM"	328/123	66.8/22 ³	52/22 ³

¹Whewhellite and/or weddellite;

²sediment from 2 ml of urine aliquot;

³p < 0.001

Table 3. Frequency (%) of the accompanying phases in urine of CaSF.

Reference	Method	Urate and uric acid	Calcium phosphate (brushite, carbapatite, and/or struvite)
Cottet and Vittu (1953)	"indirect LM"	16.8	18.5
Hallson and Rose (1976)	"indirect LM"	-	33
Werness <i>et al.</i> (1981)	PM	11	100
Caudarella <i>et al.</i> (1986)	PM	5	10
Klepikov <i>et al.</i> (1991)	"indirect LM"	3.6	1.2
Chevalier <i>et al.</i> (unpublished)	"direct LM"	8.9	39.1

Table 4. Maximum sizes of CaOx single crystals and aggregates (in μm) observed in urine of CaSF and NS.

Reference	Method	Single crystals CaSF/NS	Aggregates CaSF/NS
von Sengbusch and Timmermann (1958)	"indirect LM"	60/-	700/-
Dhanamitta <i>et al.</i> (1970); Valyasavi <i>et al.</i> (1973)	"indirect LM"	80/20	500/
Hallson and Rose (1976)	"indirect LM"	25	-
Buli <i>et al.</i> (1982)	"indirect LM"	200/50	300/-
Baumann (1978); Baumann <i>et al.</i> (1984)	"indirect LM"	30/-	300/-
Klepikov <i>et al.</i> (1991)	"indirect LM"	44/17	-/12.2
Daudon <i>et al.</i> (unpublished)	"direct LM"	120 ± 16/32 ± 45 ¹	296 ± 40/88 ± 41 ²

¹p < 0.05; ²p < 0.02

Table 5. Particle aggregation ratio in CaSF and NS as observed by LM studies.

Reference	Aggregation ratio CaSF/NS (%)
Sriboonlue <i>et al.</i> (1990)	40/22
Hallson and Rose (1976)	8/2
Baumann <i>et al.</i> (1984)	75/12
Daudon (1989)	12/8

stored up to two hours at 22 and 37°C (Elliot and Rabinowitz, 1980) or in sizes and aggregation ratios of samples stored 3-4 hours at room temperature (Fazil Marickar *et al.*, 1988) were observed using LM. Using PC, a shift towards lower volume-size distribution curves was observed but only after urine storage for 3 hours at 27°C (Irvine *et al.*, 1986). When the whole urine sample was used without dilution and acid treatment, it was observed that the number of crystals changed rapidly as a function of temperature and time (Adamthwaite, 1983a,b). These variations were attributed to the numerous tiny calcium phosphates crystals that are in suspension in urine (Ahlstrand *et al.*, 1984).

Results of Cx Studies in CaSF

In this section, Cx studies in CaSF are classified by instrument family. According to the authors' own conclusions, they are further divided into "Cx proponents" and "Cx opponents" regarding interest of Cx in CaSF. The main results from the "proponents" are grouped in Tables 2-5.

LM studies

"Proponents": One of the first studies was performed on members of a military unit stationed in India (Black, 1945). The author identified two types of CaOx crystals, one with "octahedral or envelope" form and the other with a "dumb-bell or biscuit" form. These crystals were later recognized as the main habits of weddellite and whewellite, respectively. Black (1945) further noted that Cx episodes were related to oxaluria, low water intake and "a lot of strong tea" consumption. He concluded that oxalate crystals can be passed in the urine "for years without any tendency to calculus formation". Cottet and Vittu (1952, 1953) performed, apparently for the first time, comparative Cx studies between CaSF and NS after a dinner with water restriction. These studies gave the first quantitative evaluation of Cx frequencies both in CaSF and NS for "all particles", CaOx and accompanying phases (Table 2). First attempts to study particle sizes and number using LM were performed by

studying the particles sieved from urine samples on filters with several mesh sizes. (von Sengbusch and Timmermann, 1957a,b, 1958). It was shown that intake of food-stuffs with a high oxalate content causes increased excretion of "microstones", i.e., large crystalline aggregates with sizes up to 200-700 μm in conjunction with single crystals. In studies performed in Thailand (Dhanamitta *et al.*, 1967, 1970; Valyasavi and Dhanamitta, 1974, Valyasavi *et al.*, 1967, 1973) where children living in rural areas suffer from bladder lithiasis, CaOx crystals were "commonly found" in urine. A dietary survey showed that children in villages were eating local vegetables rich in oxalate, contrary to children in urban areas. Results of studies performed between children eating oxalate-rich vegetables versus control children fed with mother's milk, water and rice showed that numerous medium (10-20 μm) and large (25-80 μm) crystals as well as numerous aggregates up to 500 μm were observed with a much higher frequency in the children eating oxalate-rich foods. In Great Britain, Hallson and Rose (1976) observed an "all crystals" Cx frequency of 48% in CaSF versus 22% in NS. Aggregation of CaOx crystals occurred in 8% of urine samples from untreated stone formers compared to 2% for urine samples of NS. Hallson and Rose (1976) also performed quantitative Cx studies using PC (see below). Later, Hallson and Rose (1978) introduced an evaporation method for studying urine crystallization properties. In Germany, microphotographs of urine sediments were taken after giving an oxalate oral dose to 18 recurrent active CaSF and 16 NS (Baumann, 1978). It was shown that 9 patients formed aggregates with diameters above 50 μm in the urine against only 1 subject in the NS group. More recently, Baumann *et al.* (1984) studied 12 idiopathic recurrent CaSF and 16 NS after oxalate ingestion. A 140 μm maximum size for weddellite crystals was observed in CaSF against 30 μm for NS. Similarly, Cx frequency was 25% in NS against 80-90% in CaSF after oxalate ingestion. All groups responded to oxalate loading with a considerable increase in whewellite particles.

All the preceding studies were performed using "indirect LM", i.e., on urine particles isolated after several technical steps. In the last few years, "direct LM" studies of the urine particle were initiated in France by Daudon and his co-workers (Daudon, 1993; Daudon and Réveillaud, 1984; Daudon *et al.*, 1983, 1987a,b, 1989; Nguyen *et al.*, 1987). Cx particle parameter studies were performed using first urine samples of the morning collected at room temperature and examined in the two hours following voiding. These studies have been performed in a continuously growing patient data base currently composed of 328 CaSF and 123 NS. The main results concern the frequency of the various crystalline phases, sizes and aggregation ratios. The relationship

between whewellite and oxaluria was confirmed and the respective role of calcium and oxalate urinary concentration on the control of CaOx phases were analyzed. It was found that the voided CaOx molecular phases depend on the calcium/oxalate molar ratio with weddellite and whewellite particles nearly exclusively present for calcium/oxalate ratio values over 14 and under 5, respectively. On the contrary, CaOx Cx frequency is dependant on the urinary calcium oxalate molar concentration product (Daudon and Réveillaud, 1984; Daudon *et al.*, 1989). In a recent Russian study of 372 nephrolithiasis patients, Klepikov *et al.* (1991) found urinary crystals in 44% of the patients against 17% of "symptomless subjects"; accordingly, they suggest consideration of Cx "as a preclinical manifestation of urolithiasis".

"Opponents": One of the most cited reports in the field of Cx studies in CaSF is from Dyer and Nordin (1967) who studied urine samples from 10 male hypercalciuric stone-formers; these urine sample were kept at 37°C "until crystals were observed". The only crystals which were observed were calcium oxalate and "one form of calcium phosphate". There were "no clear difference between stone-formers and controls" with regard to the voided crystalline phases. Elliot and co-workers (Elliot and Rabinowitz, 1976; Elliot *et al.*, 1976) studied 122 hospital urine samples from patients with no evidence of past stone disease. They used light and polarized microscopy and studied crystal molecular phases, habits and sizes. The two main weddellite habits, the bipyramids and the dodecahedral forms and the whewellite habits, with oval and dumb-bell forms, were recognized. Particle sizes were shown to be from 2 to 22 µm for whewellite and 2 to 40 µm for weddellite. In another study, Elliot and Rabinowitz (1980) studied crystals size by structure and habit in urine samples from 27 NS and 22 SF and found that "the mean crystal size did not support the conclusion that SF excrete larger crystals than normal subjects". They concluded that "there was no correlation between crystalluria and severity and duration of disease". Winkens *et al.* (1988) studied the urinary sediments from 148 SF and 144 NS and found that CaOx was the "predominant" crystal phase but found no difference between SF patients and NS for particle frequencies, numbers or sizes; they concluded that this examination is "without clinical value".

PM studies

"Proponents": Smith and colleagues (Smith, 1976; Van Den Berg *et al.*, 1976) developed PM studies in lithiasic patients. In the first series, they studied 8 patients with primary hyperoxaluria and 14 matched normal subjects. The frequency of crystalluria was 91% in patients against 18% in NS; the crystals were whewellite with sizes up to 100 µm "with evidence of aggregation".

The best documented PM study is by Werness *et al.* (1981) in which 2524 voidings were studied: 202 urine samples from 16 NS, 686 samples from 47 primary hyperparathyroidism patients, 182 samples from 12 primary hyperoxaluria and 1454 samples from 89 idiopathic CaSF. Crystal numbers voided during 3 consecutive 24-hour periods were quantified using a "semi-quantitative score" as described in **Particle number** under **Petrographic microscopy** section. It was shown that all patients, and CaSF in particular, had significantly greater crystalluria scores than NS ($p < 0.001$). CaOx molecular phases appear to be under the control of oxaluria, and patients with primary hyperoxaluria had 100% of voidings with major or only whewellite crystals. In idiopathic CaSF, weddellite was 45% against 8% whewellite. Surprisingly, whewellite was observed in 18% of NS but no weddellite. Buli *et al.* (1982) studied Cx parameters including crystal number, sizes, and number of aggregates in 50 CaSF; these variables increased significantly in CaSF as compared to NS. More recently, Caudarella *et al.* (1986) studied 86 recurrent CaSF and 40 NS. It was observed that Cx was composed of weddellite "most frequently" in CaSF, whewellite being found less frequently (9%) whereas weddellite was "nearly exclusively" found in Cx of NS.

"Opponents": In recent studies (Hermann and Schwille, 1992; Hermann *et al.*, 1991), 78 male and 38 female idiopathic recurrent CaSF were studied. Unexpectedly, CaOx crystals were either never or "only sporadically" seen in patients with pure CaOx or mixed CaOx-phosphate stones. Hermann and Schwille (1992) concluded that "calcium oxalate crystalluria does not qualify as a diagnostic aid".

PC studies

"Proponents": The use of PC for Cx studies was launched in 1969 and the method has been extensively applied (Robertson, 1969; Robertson and Peacock, 1972; Robertson *et al.*, 1969, 1971, 1974, 1976a,b). Idiopathic recurrent CaSF and NS in the same conditions of diet and fluid intake were studied. A higher "volume-concentration" of crystals (mm^3 crystal/ml urine) was noted in CaSF. This increase was attributed to a greater average crystal size in CaSF and not to the particle number. Crystals excreted by controls were small (3-4 µm in diameter) with little or no aggregates, whereas, recurrent SF passed, in addition to small crystals, larger crystals (10-12 µm) as well as polycrystalline aggregates (from 20 to 300 µm). Robertson *et al.* (1972) refined the particle size-distribution relationship and showed that "whereas the normal control excreted his calcium oxalate as a unimodal distribution of small particles, the stone-former had in addition a second peak of much larger particles". In addition to a peak centered around 7-8

μm , a second peak of much larger particles (20-40 μm in diameter) was found in CaSF. Using a dividing line set at 12.2 μm , an oral load of oxalate given to a CaSF resulted in the appearance of a dramatic second peak, contrary to NS who showed no increase. Using LM, the larger particles were identified as weddellite crystals and were often found to occur in aggregates up to 200 μm in diameter. The same authors studied the relation between the percentage of large crystals and aggregates of calcium oxalate with sizes arbitrarily set as 12 μm with an index taking into account both urine CaOx supersaturation and inhibitory activity. The study was performed in 8 recurrent idiopathic CaSF and 8 NS. The results show that the percentage of "large crystals" is positively correlated with a "saturation-inhibition index" (Robertson *et al.*, 1976a). Finally, a linear relationship was found between the average percentage of large CaOx crystals and aggregates excreted by CaSF patients and the severity of the disease (Robertson *et al.*, 1981). Following these studies, a number of laboratories performed similar PC studies in CaSF. In Great Britain, Hallson and Rose (1976) found: "agree in the main with these findings although there were striking exceptions". In the United States, studies conducted by Crassweller *et al.* (1979) using Robertson's method (not cited) gave essentially the same results with both the particle volumes and sizes significantly higher in CaSF. Recently, in a study of Cx in stone-forming runners, it was reported that in stone formers, the volume-size distribution curve is trinodal with 3 peaks occurring respectively for particle diameters of 2.5, 9 and 25 μm , contrary to NS who present only the first peak (Rodgers *et al.*, 1991).

"Opponents": Hartung and Leskovar (1976) studied an indefinite number of NS and CaSF using the Coulter counter technique (Robertson, 1969). There was no significant difference in crystal number and crystal volume between SF and controls. In another study, the poor reproducibility of the PC size-spectrum results led the author to stress that this examination is "of little use" (Baumann, 1978).

Cx Studies in CaSF: What are the Common Results?

Despite many technical and clinical differences, a number of studies agree that several Cx parameters appear significantly different between CaSF and NS. A first series concern the particle phase composition and their relative frequency. But, the most cited parameter is the frequency of "all particles" always higher in CaSF as compared to NS (Table 2). This parameter alone underlines the fact that CaSF are more prone to void crystalline particles than NS. When the relative frequencies are studied as a function of the crystalline phases, wed-

dellite is the most often observed phase in CaSF; it is found 2 to 4 times more frequently than in NS (Table 2). Phosphate and urate particles are also observed in CaSF contrary to NS (Table 3). Amongst the quantitative particle parameters, the most common results relate to the particle size. Using PC, "proponents" agree that CaSF voided particles are of larger sizes which can be grouped in a peak for sizes greater than 12.2 μm . Using LM, particle sizes up to 300 μm or more are observed (Table 4). Another parameter of interest might be an increased particle aggregate formation ratio (Table 5). In summary, most "proponents" of Cx studies in CaSF agree that CaSF appear more likely than NS to present Cx episodes; the crystalline phases are usually not observed in NS. These particles are often of larger sizes and are in an increased aggregation state. These observations favor Cx examinations in these patients. They also involve important practical consequences regarding the methods, instruments, and protocols used for Cx studies in CaSF.

Discussion

Cx studies are not performed in most urological centers. This is largely due to technical difficulties linked with this examination. A relevant Cx method should be able to: 1) perform spontaneous Cx studies, i.e., to comply with requirements to retain, as near as possible, urinary particles in their state at voiding; 2) allow a study of the largest range of qualitative and quantitative particle parameters; and 3) be compatible with routine clinical applications in terms of simplicity, speed and associated costs. In this section, we attempt to outline such a method for the two main technical steps of Cx study, namely urine sampling and instrumental analysis. We finally examine the requirements for Cx protocols in CaSF.

Which method for urine sampling, storage and handling?

Urine sampling: Cx studies can be performed on early morning as well as on random urine samples that are easier to collect (Crassweller *et al.*, 1979; Fazil Marickar *et al.*, 1988; Hallson and Rose, 1976). Nevertheless, due to the overnight water restriction period, first urine samples of the morning are probably a better indicator of the calculogenic property in CaSF and should be favored as far as possible (Daudon, 1993).

Urine storage: Storage periods of less than two hours at laboratory temperature give reliable results while at the same time being more convenient for patients and for laboratory work. Nevertheless, due to large temperature variations observed in some countries, laboratory temperatures during summer may be pretty

high and appropriate measures are required for storage of the samples. As pH variations in infected samples will modify Cx particle parameters, pH of the urines must be recorded just after voiding and then at the time of study (Daudon *et al.*, 1983; Dyer and Nordin, 1967).

Urine handling: The less demanding the sample preparation, the more possible it is to examine particles in their native state. Although there has been no comparative study of the effects of filtration, centrifugation or drying steps, these time-consuming and costly operations are likely to affect the particles. In addition, as observed during the study of cells in urine sediments, methods based on the study of sediments are likely to give inaccurate results with poor repeatability (Gadeholt, 1964; Winkel *et al.*, 1974).

Which instrument for particle study?

Particle counting (PC): PC does not allow the study of particle qualitative parameters and is restricted to the quantitative study of particles. Particle counters are standardized using spherical beads whereas most urinary particles are far from being spherical (Figures 1 and 2). It has been argued that the particle size-distribution spectra obtained by PC do not put an equal emphasis on the counting of all particles regardless of their sizes (Ryall and Marshall, 1978). Regarding particle quantity deduced from the size-distribution number transformed into volume, this can result in errors due to the cubic-size relationship involved (Parsons, 1992; Ryall and Marshall, 1978). When the number and the size of crystals in urine samples were measured by "direct LM" and PC, large discrepancies were found between the results of the two methods (Hennequin *et al.*, 1991). Finally, for particle identification, another method such as LM (Hallson and Rose, 1976; Robertson and Peacock, 1972; Robertson *et al.*, 1974) or SEM (Rodgers *et al.*, 1991) must be used.

Petrographic microscopy (PM): PM does not allow crystal phase identification of all particles (Hermann and Schwille, 1992; Hermann *et al.*, 1991). Particle size study is not performed and particle abundance is given using a "crystalluria score", "a gross quantitation of the degree of crystalluria" (Werness *et al.*, 1981). PM requires skilled observers not usually found in most clinical centers.

Light microscopy (LM): LM is well suited for particle frequency and particle qualitative studies such as crystalline phases and crystal habit studies. Regarding quantitative aspects, particle size measurements can be performed on all particles except for those particles whose sizes are less than 1 μm , i.e., below the instrument resolution limit. The largest particle sizes can be recorded for each phase. In order to have a more repre-

sentative evaluation of the particle size-distribution spectrum, we define the particle "mean size" as the mean of the largest dimensions of the particles belonging to the same crystal phase measured in at least twenty different optical fields (Daudon, 1987a). Particle concentration and number are computed by taking into account the slide chamber volume. An estimate of the particle volume can be computed for the main crystal habits using an unit particle volume by assimilating the particle to the nearest simple geometrical volume. This method has been useful for the follow-up of patients in whom large variations in the volume of the same voided crystalline phase occurred (Jouvet *et al.*, 1994). Concerning sample preparation, most LM studies are performed using "indirect LM" on sediments. "Indirect LM" suffers from the same drawbacks as PC and PM with regard to the risks for native particle properties, the inaccuracy related to work on sediments, and the time and costs associated with particle preparation. Thus, among all methods used for Cx studies in CaSF, "direct LM" appears the best suited in terms of simplicity and efficiency for all steps of Cx examinations.

Which Cx protocol for Cx studies in CaSF?

Using various Cx protocols and techniques, it has been shown that Cx can be reduced as a result of drug and diet treatments (Buli *et al.*, 1982; Caudarella *et al.*, 1986; Cottet and Vittu, 1953; Crassweller *et al.*, 1979; Daudon *et al.*, 1987a; Hallson and Rose, 1976; Hallson *et al.*, 1976; Robertson *et al.*, 1974, 1976b; Smith, 1976; Van Den Berg *et al.*, 1976; Valyasevi *et al.*, 1967, 1973; Werness *et al.*, 1981). These studies were conducted on the simple premise that "if an antistone therapy stops crystalluria, it can be expected to retard urolithiasis" (Werness *et al.*, 1981). Using multivariable statistical studies, we showed that Cx is the first discriminant variable between CaSF and NS (Chevalier *et al.*, in preparation). These results show that Cx is a primary significant risk factor in the calcium stone disease and the value of Cx studies and attempts for its control in CaSF is well founded.

The question still remains: which Cx protocol must be used in order to apply Cx for the follow-up of this disease? Obviously, the study of a few samples over a short time period is likely to give results without great meanings in most patients for whom no etiologic factors can be clearly identified (idiopathic CaSF) (Nordin, 1979). Cx protocols must be used which are able to take into account the variability of Cx due both to biological as well as to technical factors (see **Urine Sampling and Processing** above). Repeated Cx observations are needed in order to define a patient's "specific Cx status", i.e., his Cx frequency, the compositions, sizes, aggregation ratios of the voided particles, etc.

But, how long and how often must Cx examinations be performed in order to give results taking into account Cx variability in a patient? Three periods can be distinguished when deciding to use Cx examinations in CaSF. In the first period, Cx characteristics are determined and the study is conducted in the absence of treatments as far as possible. Next, Cx examinations are used to assess the efficiency of a medical treatment, including diet and drug treatment, in controlling Cx. After having successfully determined these conditions, Cx will be performed during the patient follow-up. In order to answer the question of the frequency of Cx examinations during these periods, comparative and prospective studies are needed. It can only be expected that during the follow-up period, the frequency of Cx examinations will be less than in the first two periods in as much as no change in the medical status and/or living habits of the patient are expected. In any case, many Cx examinations are needed, and the possibility of obtaining rapid and reliable results using "direct LM" as suggested above is of further interest.

Conclusions

As early as in the first study analyzed, it was noted that crystals can be passed in urine "for years without any tendency to calculus formation" (Black, 1945). Later studies have shown that Cx in CaSF is more frequent and is composed of crystalline phases rarely observed in NS which tend to form larger particles and aggregates. Furthermore, a number of studies have shown that Cx can be modified as a result of a medical treatment. Thus, Cx examinations are strongly advisable in these patients at all stages of the disease. Unfortunately, largely due to technical problems, there is at present no consensus for the follow-up of CaSF using Cx. Analysis of each technique shows that "direct LM" is a simple and efficient method. Use of this method in long term comparative studies could be of value to further assess interest of Cx examination based protocols, in particular, for the prevention of stone recurrence in these patients.

Acknowledgements

This study was financially supported by the "Caisse Nationale d'Assurance Maladie des Travailleurs Salariés" CNAMTS, Sécurité Sociale, France: grant number 701039.

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Discussion with Reviewers

R. Tawashi and M. Akbarieh: From your experience, what is the most critical variable in writing a protocol for Cx studies using LM?

Authors: There are several critical variables which have to be fulfilled in order to perform reliable Cx studies in CaSF. The first critical variable is to study the urinary crystalline particles in a state as near as possible to voided, i.e., to perform "spontaneous" Cx studies. It should be noted that this requirement is applicable irrespective of techniques and instrument used. However, taking into account the numerous handling steps required by all other methods including "indirect LM", only "direct LM", which does not require any urine preparation steps, is able to fulfill, at best, the conditions of "spontaneous" Cx studies.

Two other requirements are also independent of the chosen Cx technique: the need to perform repetitive "spontaneous" Cx studies and the need to select a set of reference Cx parameters. Due to patient as well as healthy subject variability, only repetitive studies can define the qualitative and quantitative Cx parameters with a statistical value: molecular phases voided and their frequencies, particle sizes, aggregation ratio, etc. Obtaining one or even a few Cx results in a patient is likely to have, in most cases, only no more than an anecdotic value in particular for idiopathic calcium nephrolithiasis patients. The third critical variable is linked to the need of having a reference set of Cx parameters. One must choose between basing this reference set on a strictly defined patient cohort or on the patient himself. Due to the difficulty in controlling all parameters (age, sex, calcium nephrolithiasis etiology, stage of the disease, regimen, diet, drugs, etc.), using the patient as his or her own reference is often the most accurate and practical way for using Cx studies in patient follow-up. Only in the case of large comparative studies, can sever-

al patient series be used. We take this opportunity to stress that in most cases, statistical requirements are such that the number of patients and urine samples required, combined with the long time associated with most recurrent stone events, strongly appeal for cooperative studies between stone clinics on a national, or when possible, an international basis.

R. Tawashi and M. Akbarieh: In your view, what would be the role of crystal phase transition in Cx determination or the interpretation of results obtained, particularly when it comes to the storage and/or filtration of urine?

Authors: As strongly advocated in our paper and repeated above, we think that clinically meaningful Cx studies must be "spontaneous" Cx studies. Other Cx results need careful evaluation as to whether the results of Cx changes are the consequences of particle preparation steps such as filtration, storage, dilution, etc., or are they the results of physicochemical steps such as cooling, pH adjustments, etc. Nevertheless, well-controlled physicochemical treatments are easier to interpret than the variations of Cx parameters due to the handling steps. We have some experience on the modifications of Cx parameters of urine samples after 48 to 72 hours storage at 4°C. We always perform these studies after having first studied the urine samples immediately after voiding, i.e., their "spontaneous" Cx parameters. Results of our studies (unpublished) can be summarized as follows. The main interest of crystal phase transitions observed after urine storage in the cold is the study of the relationships between crystal phases and the biochemical composition of urine. Depending on the urine supersaturation level, crystalline phase transitions will occur more or less rapidly. For instance, when urine pH is low, namely less than 5.2, uric acid dihydrate crystals can form in urine despite low urate concentration (1-1.5 mmol/l). This crystal phase may be either stable or able to transform into anhydrous uric acid crystals depending on the supersaturation level. In contrast, if urine pH is higher than 5.5, precipitates of complex amorphous urates rather than uric acid dihydrate may occur in cases of high urate concentration (> 4 mmol/l). In such conditions, the formation of uric acid dihydrate crystals will occur progressively at the expense of disappearing amorphous urates.